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An Exosomal mRNA Urine Test for Detection and Risk Stratification of Human Kidney Transplant Rejection

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Introduction: We recently discovered 2 urinary exosomal mRNA signatures to identify and differentiate T-cell–mediated rejection (TCMR) from antibody-mediated rejection (ABMR) in kidney transplant recipients. Here, we developed Exosome Transplant Rejection Urine (ExoTRU), a urinetest based on a 4-gene signature from the previous discovery cohort, showed its clinical utility in a new cohort of kidney transplant recipients undergoing clinically indicated biopsies, and validated it through a separate laboratory in an independent-cohort of patients.

Methods: A workflow suited for clinical laboratories was developed, allowing for smaller urine volumes and widely standardized qPCR instrumentation. A total of 226 urine samples from 214 patients were paired with clinically indicated biopsies. Urinary exosomal mRNAs levels were evaluated for previously defined targets.

Results: Four mRNAs (IL32, B2M, CXCL11, and PGK1) performed well in distinguishing biopsies with rejection or significant inflammation from those without inflammation, achieving 94% sensitivity, 62% positive predictive value, and 52% specificity. Patients who tested positive by the signature but negative by biopsy were nearly twice as likely to experience adverse outcomes in the 5-year follow-up period, including subsequent rejection, thereby showing the limitations of kidney biopsies and the prognostic potential of molecular signatures. The evaluation of an independent validation cohort showed similar performance, achieving an area under the curve (AUC) of 0.838. Another 6-gene signature distinguished TCMR from ABMR, with an AUC of 0.756.

Conclusion: Exosomal mRNA gene signatures identified patients with different stages and classes of rejection, including early stage and significant inflammation, enabling improved decision-making and patient management and reducing unnecessary biopsies by 45%. This represents a potential tool for risk stratification based on poor outcomes in patients with positive signatures.

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Chronic kidney disease is a growing global health concern[1] and impacts nearly 15% of the US adult population.[2] Ultimately, a continuous decline in renal function necessitates dialysis or kidney transplantation for survival.[3] Transplantation is the preferred treatment for patients with chronic kidney disease who

progress to end-stage renal disease and corresponds to increased life expectancy.[4,5] In the US, there are > 250,000 living kidney transplant recipients with functioning grafts.[6] The incidence of acute rejection ranges from 10% to 20%, as reported by major registries from the US and Australia/New Zealand.[7] Approximately 7% of patients experience acute rejection within 1 year after transplantation—with rates of subclinical rejection up to 20%—which remains one of the main predictors of long-term allograft loss.[6]

The gold standard for diagnosing acute rejection involves tissue biopsy assessed using the Banff classification scheme.[8-10] However, biopsies are invasive, linked to complications and hospitalization, and are constrained by interobserver variability and sampling error.[11,12] Renal transplant function is assessed through serum creatinine and proteinuria. Nevertheless, these markers are delayed indicators of rejection and lack both sensitivity and specificity.[13] Despite substantial efforts to identify novel biomarkers for acute rejection—such as urinary and serum metabolomics, proteomics, and genomics—none have demonstrated sufficient sensitivity and specificity, particularly for early-stage rejection (TCMR1A), which is predominant in the first year posttransplant.[14-16] There is a significant unmet need for developing noninvasive biomarkers to diagnose acute and chronic rejection, help with therapeutic decision-making, and improve survival in transplant recipients. Molecular diagnostics that identify pathophysiological processes developing in the graft itself have the potential to identify rejection even before any injury occurs.

Urinary exosomes are spherical extracellular vesicles surrounded by lipid bilayers ranging in size from 50 to 200 nm that carry RNA from donor cells, offering a source of robust biomarkers.[17,18] Exosomes are also referred to as extracellular vesicles; however, in this study, we utilized the exosome nomenclature for any urine extracellular vesicle < 0.8 μm in diameter. Interest in utilizing exosomal RNA for diagnostics has grown rapidly in recent years, and its diagnostic applications span a wide range of diseases. Identification of cancer-specific mutations on exosomal RNA from biofluids was first shown in 2008,[19] and the first commercial urine-based exosome RNA test was launched 8 years later in the form of a prostate cancer test.[20] Recent studies examining the composition of urinary exosomes secreted from different segments of the nephron reveal insights into renal physiology and disease-driven kidney dysfunction.[21] We previously determined that patients with acute rejection had higher levels of $CD3^+$ urinary exosomes, reflecting T-cell infiltration in renal allografts.[22] Importantly, nucleic acids are contained within the exosomal vesicle, providing protection from degradation compared with other freely circulating molecules, and a more robust diagnostic and prognostic signature with the potential of identifying graft-specific immunological processes in the posttransplant setting.

Previously, we built on the success of utilizing exosomal mRNA in the ExoDX prostate test,[20,23,24] and we developed a urine-based exosomal mRNA assay ExoTRU that discriminates acute rejection from no-rejection.[25] In this paper, we migrated our urine test, ExoTRU, from an open array to a custom TaqMan assay using real-time PCR widely available in clinical laboratories. We optimized the protocol for smaller urine volumes and analyses on the same day. Exosomal mRNA from urine is stable when stored at 4 °C, allowing for easy collection and storage in clinical settings.[25]

We further show that our new 4-gene signature is sensitive in detecting any-cause rejection and significant underlying kidney inflammation, and we have been able to show that a positive signature holds high predictive performance for subsequent adverse allograft outcomes. In addition, we transferred the methodology to an independent laboratory to evaluate its performance in a distinct independent validation cohort.

METHODS

Patient and Sample Information

Patients were enrolled from 4 renal transplant centers. The study was approved by the institutional review board of each site, and the patients provided written informed consent in accordance with the Declaration of Helsinki. All kidney transplant recipients who underwent clinically indicated kidney allograft biopsy were enrolled. Urine samples were collected within 24 hours of biopsy and before the initiation of any rejection treatment. To ensure they reflect real-world diagnoses, all the biopsies were included, inclusive of biopsies with significant inflammation, such as BK virus–associated nephropathy (BKVAN) and recurrent glomerulonephritis. To validate the transferability and standardization of the signature, we used an independent validation cohort comprising samples from patients with clinically indicated biopsies and patients who were considered long-term stable. Patients were classified as long-term stable if they remained free from acute or chronic rejection since transplantation, had no proteinuria, and had not experienced more than a 20% decrease in the estimated glomerular filtration rate (eGFR) 6 months before enrollment.

Donor demographics, including age, sex, race (as identified in the electronic health record), and clinical characteristics were collected from the respective electronic health record systems. Patient biopsies were evaluated according to the established protocols for each site. The renal transplant biopsy specimen pathologist reports and the Banff classification[26] were used to discriminate any-cause rejection (including TCMR Grades 1A, 1B, 2A, 2B, and 3), borderline (BL) rejection, active ABMR, and chronic active ABMR from no-rejection status. The eGFR and change in eGFR were calculated using the Modification of Diet in Renal Disease equation with standardized serum creatinine values.[1]

Exosome Isolation, mRNA Extraction, and Gene-Expression Profiling

Voided urine samples were collected within 24 hours of the biopsy, and before any rejection treatment, and stored at −80 °C. Samples were thawed and 3 to 10 ml of urine was centrifuged at 2000*g* for 20 minutes to pellet the cells and cellular debris before extraction. The exosomal RNA was isolated using a proprietary exosome isolation kit (ExoLution RNA; Exosome Diagnostics, Waltham, MA). RNA was eluted in nuclease-free water and reverse-transcribed using a VILO cDNA synthesis kit (Thermo Fisher, West Hills, CA).

Target mRNAs comprised our previously identified and published 15-gene signature to discriminate any-cause rejection and a 5-gene signature to differentiate TCMR from ABMR.[2,5] The union of these 2 signatures formed our candidate gene set of 18 mRNA targets (*B2M*, *BMP7*, *C3*, *CD44*, *CD74*, *CXCL11*, *CXCL14*, *IFNAR2*, *IFNGR1*, *IL18BP*, *IL32*, *IRAK2*, *NAMPT*, *PYCARD*, *SERPINA1*, *STAT1*, *TBP*, and *PGK1*). These 18 mRNA assays were migrated from the original discovery platform, the TaqMan OpenArray real-time PCR system (Thermo Fisher Scientific, West Hills, CA), to custom-designed assays. Specific mRNA targets were preamplified (Supplementary Methods) and the custom-designed assays were validated to be equivalent to the assays used in the original publication (Supplementary Figure S1).

Outcomes Measures

Five-year follow-up data were obtained from the patients' charts of a subset of patients with a negative biopsy kidney and a positive urinary exosome signature. The composite outcomes included a $>$ 30% decrease in eGFR, subsequent rejection, *de novo* donor-specific antibody, death, or loss of graft with either return to dialysis or retransplantation. Follow-up began immediately after the kidney biopsy or urine sample collection date until the earliest outcome. A $>$ 30% decrease in eGFR was calculated in comparison with the baseline eGFR reported in patient records using the MDRD equation.

Statistical Analyses

Raw Ct values for all qPCR assays were filtered for quality control and normalized to the endogenous control target, *PGK1*. Imputation was performed for mRNAs with missing values or Ct values that exceeded the lower limit of quantitation for the assay, as defined during assay verification. For classifier development, models, hyperparameters, features, and threshold selections were performed under 5-fold 2×-repeated cross-validation, grouped by donor (Supplementary Methods).

Clopper Pearson confidence intervals were calculated for the classifier performance metrics, including sensitivity, specificity, positive predictive value, and negative predictive value. DeLong's test was used to determine differences in AUC between 2 classifiers.[3] Differences in classifier score distributions between nonrejection cases and different rejection subtypes (TCMR 1A, TCMR $\geq$ 1B, and ABMR) were determined via 2-sided Mann-Whitney U rank test. Cox proportional hazards regression was performed according to a 1-sided significance test to assess prognostic signals.

RESULTS

Patient Characteristics

We collected 190 urine samples from 178 patients with matched biopsy specimens to facilitate urinary exosomal mRNA profiling and signature development (training cohort; Figure 1). Of the 190 samples, 121 were determined to be rejection-negative by biopsy and 69 were rejection-positive, resulting in an overall all-cause rejection prevalence of 36.3%. The baseline characteristics of the training cohort are presented in Table 1. Of the rejection-positive biopsies, 23 were ABMR (6 mixed, 5 active ABMR, 8 chronic ABMR, and 4 ABMR with BL rejection) and 46 were TCMR. Of the TCMR cases, 23 were categorized as TCMR 1A, 11 as TCMR 1B, 3 as TCMR 2A, 1 as TCMR 2B, 4 as BL, and 4 as chronic TCMR. Eight of the 69 rejection-positive samples were annotated as BL rejections (Supplementary Figure S2). The higher representation of TCMR in this cohort (67% of any-cause rejections) reflected the first 12 months posttransplant[4] and early stage rejections.

An additional 36 patients were included to independently validate the any-cause rejection signature (Validation Cohort, Figure 1, Table 2). Of the 36 samples, 22 were determined to be rejection-negative by biopsy ($n = 12$) or were obtained from patients determined to be long-term stable ($n = 10$). The remaining 14 samples were positive for any-cause rejection by

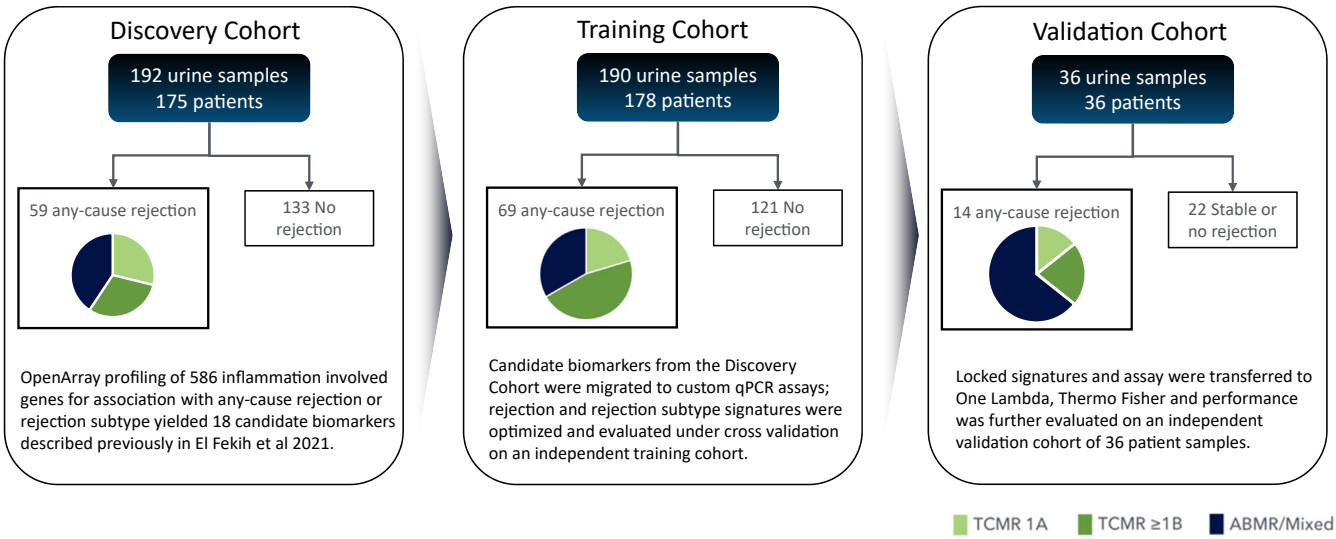

Figure 1. Urinary exosome mRNA signature development and evaluation cohorts. Urinary exosome mRNA signatures comprising 18 candidate biomarkers were identified in the Discovery Cohort presented in previously published work (El Fekih *et al.*, 2021). In the current study, the candidate biomarkers were migrated from TaqMan OpenArray designs to custom qPCR assays and optimized on an independent Training Cohort of 182 of 190 urine samples (excluding 8 borderline rejection cases). To increase the representation of immune quiescent cases during the any-cause rejection classifier optimization, we included a supplementary cohort (not shown) of 172 urine samples collected from individuals with protocol biopsies (not clinically indicated) that were negative for rejection. The any-cause rejection signature was evaluated under cross validation exclusively on the 182 for-cause samples to assess performance in the clinically indicated setting. The assay protocol and optimized classifiers were transferred to One Lambda, Thermo Fisher and performance for the any-cause rejection signature was further evaluated on an independent 36 patient Validation Cohort. ABMR, antibody-mediated rejection; TCMR, T-cell-mediated rejection.

biopsy and included 2 TCMR 1A, 1 TCMR 1B, 1 TCMR 2A, 1 TCMR 2B, 4 mixed, and 5 chronic ABMR.

Urine Exosome mRNA Signature for Any-Cause Rejection in Clinically Indicated Biopsies

Of the 190 biopsies, 8 were annotated as BL rejections and were excluded from the primary analysis and classifier development. The remaining 182 samples were used to select a classification strategy that distinguished between any-cause rejection and nonrejection cases. We selected a 4-gene (*IL32*, *B2M*, *CXCL11*, and endogenous control *PGK1*) classifier that accurately distinguished any-cause rejection from nonrejection, based on independent histopathological assessment of concomitant biopsy results. Details on the features used for mRNA selection are provided in the Supplementary Methods. This achieved an AUC of 0.731 (Figure 2a), which significantly outperformed the diagnostic performance of change in eGFR with an AUC of 0.512 on the same cohort (Figure 2b, $P = 0.00048$). The classifier achieved a negative predictive value of 93% (Table 3). Only 4 of the 61 samples annotated as rejection-positive by biopsy were classified as negative by our signature (Supplementary Table S1). Two of these 4 patients were treated for rejection 1 and 2

Table 1. Baseline characteristics of the training cohort samples

Clinical characteristic	No-rejection ($n = 121$)	Any-cause rejection ($n = 69$)	*P* value
Age, yr	51.02 ± 14.74	51.97 ± 15.33	0.67
Sex, % female	28.1	37.68	0.2
Race, % Black	19.83	21.74	0.85
SCr at biopsy, mg/dl	1.8 (1.45–2.34)	2.1 (1.6–2.69)	0.2
eGFR, ml/min per 1.73 m^2	38.73 (29.63–48.86)	31.59 (24.86–42.58)	0.02
Deceased donor, %	42.98	56.52	0.1
Time to biopsy, d	184.0 (54.0–1478.0)	395.0 (103.5–1540.0)	0.63
Thymoglobulin, %	57.02	72.46	0.03
DSA, %	11.57	37.68	0.0001
PRA, %	0.0 (0.0–0.94)	0.34 (0.0–4.0)	0.08

DSA, donor-specific antibodies; eGFR, estimated glomerular filtration rate; PRA, panel -reactive antibody; SCr, serum creatinine.
Data presented as frequencies, mean ± SD, and median (interquartile range). All demographic and clinical data are based on the day of biopsy and timed with the urine collection ($N = 190$).

Table 2. Baseline characteristics of the validation cohort

Clinical characteristic	No-rejection ($n = 22$)	Any-cause rejection ($n = 14$)	*P* value
Age, yr	57.59 ± 11.83	51.14 ± 16.06	0.1876
Sex, % female	36.36	50.0	0.5
Race, % Black	18.18	14.29	1.0
sCr at biopsy, mg/dl	1.35 (1.06–1.84)	2.22 (1.6–2.38)	0.0401
eGFR, ml/min per 1.73 m^2	58.0 (41.5–71.5)	36.0 (26.5–41.5)	0.016
Deceased donor, %	31.82	57.14	0.18
Time to biopsy, d	820.5 (202.5–1630.5)	818.5 (558.0–2172.0)	0.9949
Thymoglobulin, %	68.18	78.57	0.71

eGFR, estimated glomerular filtration rate; sCr, serum creatinine.
Validation cohort consists of a total of $N = 36$ patients.

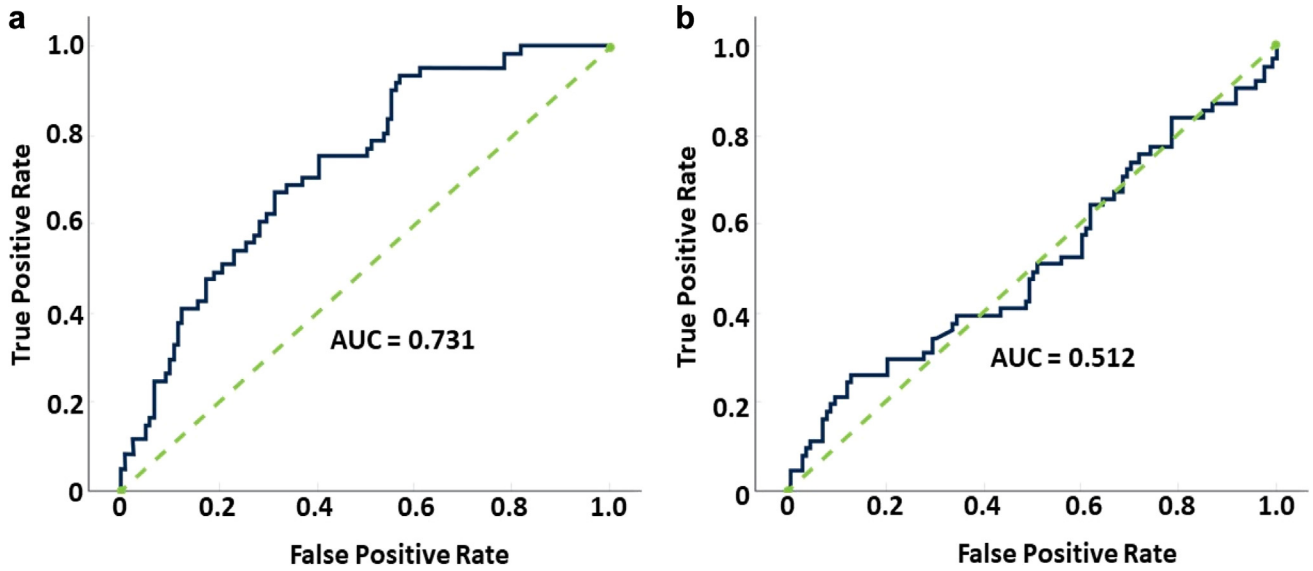

Figure 2. Urinary exosomal signature for any-cause rejection in clinically indicated biopsies. (a) Urinary exosomal signature distinguished any-cause rejection from nonrejection when evaluated over 182 clinically indicated biopsy matched urine samples, (b) significantly exceeding the predictive performance of ΔeGFR. The fraction of true-positive results (sensitivity) and the fraction of false-positive results (1-specificity) for diagnosis of any-cause acute rejection are displayed on the y- and x-axis, respectively. The AUC for mRNA signature was 0.731 and the AUC for ΔeGFR was 0.512. AUC, area under the curve; ΔeGFR, change in estimated glomerular filtration rate.

months before sample collection, whereas the biopsy of 1 patient showed features of questionable ABMR. Repeated biopsy showed rejection at the time of the false-negative urine test, which was significantly improved compared with the previous biopsy in one of the patients not receiving treatment by his provider. We also demonstrated an equivalent performance as a function of race (Supplementary Figure S3).

Next, we assessed the ability of the classifier to distinguish between distinct types of rejection and nonrejection. We found that ABMR ($P = 0.017$), TCMR 1A ($P = 0.026$), and TCMR ≥ 1B ($P < 0.001$) all scored higher than no-rejection cases (Supplementary Figure S4).

Table 3. Urinary exosomal for-cause biopsy signature evaluated on the training cohort demonstrates strong rule-out performance when considering any-cause rejection or significant underlying inflammation as positive

Diagnostic test performance metrics	Any-cause rejection only		Any-cause rejection or significant inflammation	
Metric	Point estimate	Confidence interval	Point estimate	Confidence interval
Sensitivity	0.93	(0.841–0.982)	0.94	(0.865–0.980)
Specificity	0.43	(0.34–0.523)	0.52	(0.413–0.617)
PPV	0.45	(0.364–0.543)	0.62	(0.528–0.704)
NPV	0.93	(0.827–0.98)	0.91	(0.804–0.97)
Unnecessary biopsies avoided	43%	(34–52)	52%	(41–62)

NPV, negative predictive value; PPV, positive predictive value.
Data are presented as point estimates with corresponding confidence intervals ($n = 182$ patient samples). Under the optimized threshold, the classifier applied to any-cause rejection alone maintained a sensitivity of 93%, with the potential to reduce unnecessary biopsies by 43%. When considered in conjunction with significant inflammation, the classifier achieved a sensitivity of 94% and a positive predictive value of 62%.

Discriminating TCMR From ABMR in Clinically Indicated Biopsies

We analyzed samples with any-cause rejection in the for-cause biopsy cohort to evaluate signatures capable of distinguishing TCMR from ABMR rejection subtypes. We utilized the same modeling approach (Supplementary Methods) as that used for the any-cause rejection signature and evaluated patients diagnosed with TCMR ($n = 42$, excluding 4 BL rejections) or ABMR ($n = 13$, excluding 6 mixed subtype pathologies and 4 BL rejections). We identified a 6-gene signature (*IL18BP*, *CXCL11*, *CD74*, *CD44*, *C3*, and the endogenous control *PGK1*) that accurately distinguished TCMR from ABMR, with an AUC of 0.756 (Figure 3a). We selected a threshold to rule out the ABMR subtype (targeting 80% sensitivity), achieving overall performances of 90%, 38%, 77%, and 62%, respectively (Figure 3b and Table 4).

Assessment of Inflammation and Long-term Allograft Outcomes in Clinically Indicated Biopsies Initially Negative for Rejection

Next, we examined the role of inflammation in the classification outcomes. Significant inflammation was defined as pathological lymphoproliferative infiltration, moderate-to-severe lymphocytic infiltration, interstitial nephritis (BKVAN or acute interstitial nephritis from other causes), glomerulopathy, or immune complex deposition (Supplementary Table S2). Of the 83 samples with either any-cause rejection ($n = 61$) or significant inflammation ($n = 22$), 78 were classified as positive,

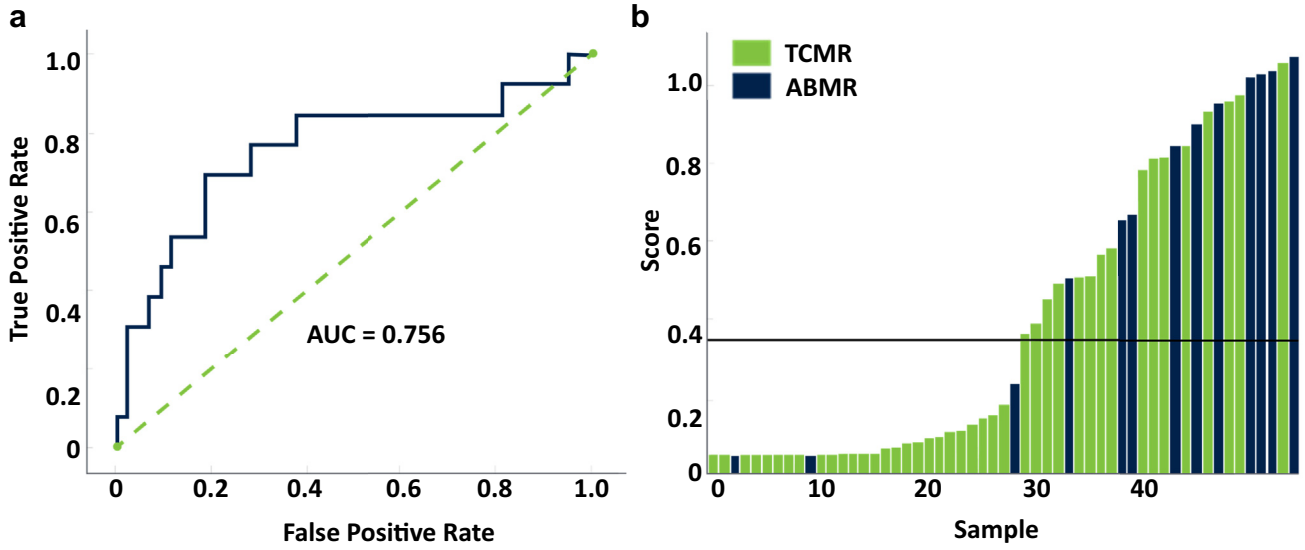

Figure 3. Urinary exosomal rejection subtype signature can accurately distinguish TCMR from ABMR. (a) Urinary exosomal rejection subtype signature accurately distinguished TCMR from ABMR with an AUC of 0.756 via patient-stratified, leave-one-out analysis. The fraction of true-positive results (sensitivity) and the fraction of false-positive results (1-specificity) for diagnosis of any-cause rejection are displayed on the y- and x-axis, respectively. (b) Waterfall analysis of urinary exosomal rejection subtype signature across any-cause rejection samples, TCMR ($n = 42$) and ABMR ($n = 13$), excluding 6 samples with mixed subtype pathologies. The black line represents the cut point to rule out the ABMR subtype, achieving an overall performance of 90% NPV and 38% PPV. ABMR, antibody-mediated rejection; AUC, area under the curve; NPV, negative predictive value; PPV, positive predictive value; TCMR, T-cell-mediated rejection.

using our model. Further assessment of negative rejection by biopsy samples identified 21 of 69 (30%) as positive by our model (false-positive) with inflammation by biopsy, whereas only 1 of the 52 rejection-negative samples classified as negative showed inflammation ($P < 0.001$, Figure 4). When considering all-cause rejection or significant underlying inflammation as positive, we achieved 94% sensitivity, 62% positive predictive value, and 52% specificity (Table 3).

Six patients with false-positive samples and no evidence of inflammation on biopsy developed rejection. This was documented on repeat clinically indicated biopsies performed 3 weeks to 6 months later for persistently elevated creatinine levels, suggesting a potential biopsy sampling error (Supplementary Figure S5). Three patients with BK viremia and no biopsy inflammation were classified as positive using our model. Two of these patients were diagnosed with BKVAN on a subsequent biopsy performed within 3 months when their kidney function failed to improve, whereas the third patient developed BKVAN 10 months later. One patient with a rejection-negative sample, no significant underlying inflammation, and who was classified as positive by our model developed relapsing BK polyomavirus-associated hemorrhagic cystitis and bladder carcinoma. All 6 cases of BK nephropathy were classified as positive using our model.

Table 4. Urinary exosomal for-cause biopsy signature to rule out ABMR relative to TCMR

Metric	Point estimate	Confidence interval
Sensitivity	0.77	(0.462–0.95)
Specificity	0.62	(0.456–0.764)
PPV	0.38	(0.202–0.594)
NPV	0.90	(0.726–0.978)

ABMR, antibody-mediated rejection; NPV, negative predictive value; PPV, positive predictive value; TCMR, T-cell-mediated rejection.
The data are presented as point estimates and their corresponding confidence intervals. The 6-gene signature cutoff point to rule out the ABMR subtype achieved an overall performance of 90%, 38%, 77%, and 62%, respectively.

To further assess the prognostic value of the any-cause rejection signature, we analyzed a subset of patients with a negative biopsy and a positive urinary signature, and follow-up outcomes available over 5 years ($n = 88$). Patients were assessed for the following composite outcomes: > 30% decrease in eGFR, subsequent rejection, *de novo* donor-specific antibody, death, or loss of graft with either return to dialysis or retransplantation (Supplementary Table S3 and Figure 5). We found that patients who received a positive score by our classifier but were rejected as negative by biopsy were nearly 2 times likely to experience an adverse event in the 5-year follow-up period than patients who received a negative score (hazard ratio =1.99, $P = 0.038$). These data highlight the limitations of renal biopsy and prognostic potential of urinary exosomal molecular signatures.

Analysis of BL Rejection Samples

When applying the classifier to BL cases, we scored 5 as rejection positives. Two of the 3 patients with BL

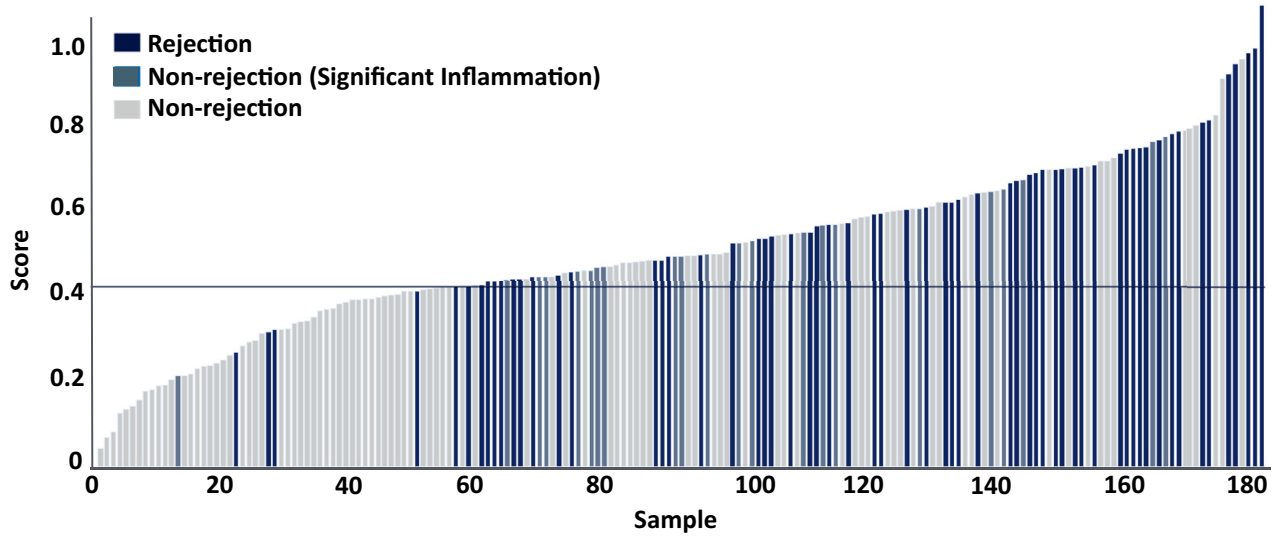

Figure 4. Analysis of urinary exosomal signature for samples with any-cause rejection or significant inflammation. Waterfall analysis of urinary exosomal signature for samples with any-cause rejection or significant inflammation. Of the 83 samples with any-cause rejection or significant inflammation, 80 were classified as positive. When assessing samples determined to be rejection-negative by biopsy, 21 of 69 (30%) classified as false positive showed significant underlying inflammation determined by biopsy, whereas only 1 of the 52 rejection-negative samples classified as negative showed significant inflammation ($P = 2e{-}^{5}$).

classified as negative by our signature did not receive treatment and remained stable for the subsequent 5 years. The third patient was lost to follow-up. Two of the 5 BL patients classified as positive had concomitant chronic antibody mediated rejection at some point posttransplantation. Another patient with BL who was classified as positive by our signature was treated with a steroid bolus. A repeat biopsy performed 5 months later showed persistent BL lesions with a positive signature at the time of repeat biopsy and declining kidney transplant function, with an eGFR of 20 ml/min per 1.73 m^2.

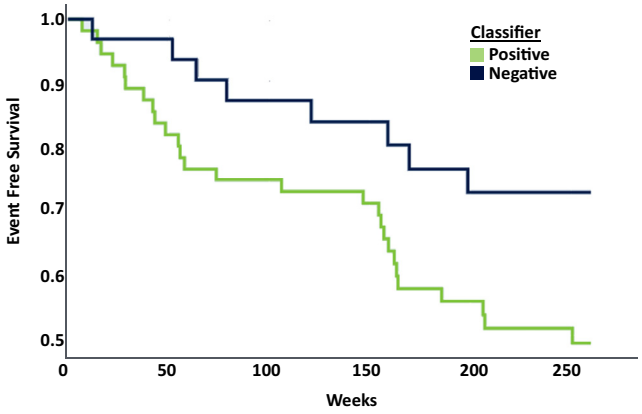

Figure 5. Analysis of urinary exosomal signature for adverse long-term allograft outcomes. Samples negative for rejection at the first biopsy were further assessed for adverse outcomes (> 30% decrease in eGFR, subsequent rejection, or loss of graft with either return to dialysis or retransplant) in follow-up data up to 5-years postbiopsy by Kaplan-Meier analysis. Patients with a positive Exo-TRU score were nearly twice as likely to experience an adverse event at the 5-year follow-up than those with a negative score (HR=1.99, $P = 0.038$). eGFR, estimated glomerular filtration rate; HR, hazard ratio.

Validation of the Urine Exosome mRNA Signature for Any-Cause Rejection

The 4-gene any-cause rejection classifier was further evaluated after transfer to an independent clinical laboratory using a distinct and independent validation cohort of 36 clinically indicated patient biopsy samples (Supplementary Table S3, Figure 6a). These results were on par with those of our training cohort, achieving an AUC of 0.838 (Figure 6b). The any-cause rejection classifier achieved a negative predictive value of 91% and a positive predictive value of 52%, while demonstrating the potential to save 45% of unnecessary biopsies (specificity) (Table 5).

DISCUSSION

In this follow-up and validation study, a positive urinary mRNA exosomal assay demonstrated the ability to identify kidney transplant recipients with acute rejection and/or significant kidney inflammation, differentiate rejection, and stratify them according to the risk of adverse outcomes within 5 years. In addition, it demonstrated a high performance as a rule-out test to avoid unnecessary biopsies. The signature was optimized and trained to detect kidney rejection, even in the early stages. The validation results indicated that it also captured other significant inflammatory events that could be beneficial in the diagnosis and prediction of long-term allograft outcomes. In the past, studies of urine and blood biomarkers have demonstrated mixed

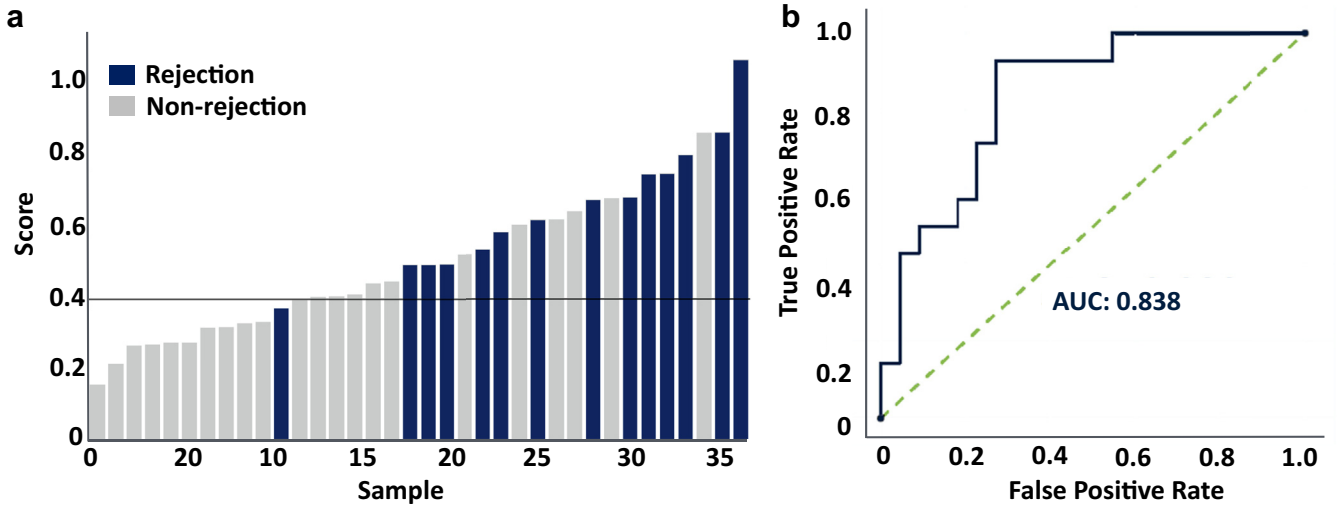

Figure 6. Urinary exosomal signature for any-cause rejection evaluated on a 36-patient validation cohort. (a) Waterfall plot of urinary exosome gene scores for the any-cause rejection signature across the 36-sample validation cohort, with matched biopsy-determined rejection status annotated in blue (rejection) and gray (nonrejection or long-term stable). The black line represents the score cutoff for the exosomal mRNA signature. (b) Corresponding ROC-curve on the 36-sample validation cohort. The fraction of true positive results (sensitivity) and the fraction of false positive results (1-specificity) for diagnosis of any-cause acute rejection are displayed on the y- and x-axis, respectively. The AUC for the ExoTRU any-cause rejection signature on the validation cohort was 0.838. AUC, area under the curve; ROC, receiver operating characteristic.

sensitivity and specificity.[27] Evaluation of urinary cell pellets launched the field of kidney transplant biomarkers, and the CTOT-04 study showed that *CD3E* mRNA, (IP-10) *CXCL10* mRNA, and 18S ribosomal RNA discriminate between biopsy samples with acute rejection versus those with no-rejection.[28] Urinary metabolomics using liquid chromatography mass spectrometry in various adult and pediatric populations have identified panels of urine metabolites with good accuracy in detecting specific allograft injuries. However, this approach remains limited by cost and the requirement for highly specialized laboratories.[29,30] Alternatively, the exploration of urine chemokines, especially CXCL9 and CXCL10, has been extensively studied as a promising biomarker for acute rejection.[31–33] More recently, donor-derived cfDNA was applied to noninvasive rejection diagnosis using a 1% threshold to discriminate patients with allograft rejection from a nonrejection subset, but failed to detect early signs of rejection, such as TCMR1a.[34]

Table 5. Urinary exosomal for-cause biopsy signature demonstrates strong rule-out performance for any-cause rejection on the independent validation cohort

Metric	Point Estimate	Confidence Interval
Sensitivity	0.93	(0.661–0.998)
Specificity	0.45	(0.244–0.678)
PPV	0.52	(0.313–0.722)
NPV	0.91	(0.587–0.998)
Unnecessary biopsies avoided	45%	(24–68)

NPV, negative predictive value; PPV, positive predictive value.
Data are presented as point estimates and corresponding confidence intervals ($N = 36$ samples). The any-cause rejection classifier maintained a sensitivity of 93%, with the potential to reduce unnecessary biopsies by 45%.

In our previous study, we identified a 15-gene signature discriminating any-cause rejection from no-rejection and a 5-gene signature to differentiate TCMR from ABMR.[25] Here, we modified the assays to custom TaqMan assays, optimized the protocol for smaller urine volumes and same-day analyses, expanded the number and diversity of samples, and increased the number of sites to accurately reflect real-world diagnoses.

We developed an optimized any-cause rejection rule-in classifier on the for-cause cohort yielding a 4-gene signature (*IL32*, *B2M*, *CXCL11*, and endogenous control *PGK1*), achieving an AUC of 0.731, which significantly outperformed change in eGFR (AUC of 0.512 in this same cohort). With a high negative predictive value and sensitivity, it demonstrates good clinical utility as a rule-out signature designed to prevent unnecessary biopsies without missing rejection-positive samples. When assessing the classifier's ability to distinguish between different types of rejection, we found that TCMR 1A, TCMR >1B, and ABMR, all scored significantly higher than the no-rejection cases. Although ddcf DNA showed low performance in diagnosing TCMR1A, our urinary signature performed well in capturing all categories, types, and severities of kidney rejection.

By applying the signature to BL T-cell rejection samples, we observed that samples classified as positive by our signature exhibited poorer outcomes upon

follow-up than those classified as negative. This finding is particularly noteworthy when considering the inherent heterogeneity within BL groups in terms of the degree of inflammation and clinical significance. This underscores the prognostic performance of our test. The assay protocols and signatures were transferred to an independent clinical laboratory and evaluated using an independent cohort of 36 samples. This study demonstrated consistent performance with the results obtained in the training cohort and highlighted the signature's generalizability to different patient cohorts and laboratory settings.

Genes comprising the any-cause rejection signature,*IL32*, *B2M*, and *CXCL11*, are mediators of inflammation. IL-32 is an intracellular proinflammatory cytokine and induces the synthesis of other cytokines, such as IL-6, TNF alpha, and IL1β.[35] Beta-2-macroglobulin is a chaperone of major histocompatibility complex class-I and plays a role in antigen presentation and immunoglobulin transport. Deleting beta-2-macroglobulin in animal models reduces immune rejection.[36] CXCL11 is a ligand of CXCR3, a receptor expressed on Th1 cells, and is an interferon gamma-induced chemokine, which is also a marker of rejection.[37] CXCL11 was reported as part of an ABMR-selective transcript in molecular studies of kidney transplant rejection[38] and found to be highly expressed in our previously published signature discriminating TCMR from ABMR.[25] Here, when assessing the role of significant inflammation on classification, we found that our 4-gene signature is sensitive in detecting any-cause rejection and significant underlying kidney inflammation. Further analysis of long-term allograft outcomes demonstrated that our signature has the potential to predict both initial biopsy outcomes and future adverse events (> 30% decrease in eGFR, *de novo* donor-specific antibody, subsequent rejection, death, and loss of graft with either return to dialysis or retransplantation) that manifest over 5 years. Patients with a negative biopsy and positive rejection signature were nearly twice as likely to experience an adverse event at the 5-year follow-up, supporting the premise that our test could be used as a tool for the risk stratification of kidney transplant recipients. However, further studies are needed to validate these observations.

Further investigation of other misclassified samples in our cohort revealed the potential influence of treatment and biopsy sampling. Two false-negative samples were obtained from patients who had been treated for acute rejection in a previous biopsy. Patients with positive BK viremia who were falsely classified as positive with our any-cause rejection signature and tested positive for BKVAN on a subsequent biopsy performed within 3 to 10 months may have biopsy sampling errors that were correctly diagnosed by the urine test.

The complexity of molecular mechanisms underlying kidney allograft rejection suggests that a combination of multiple biomarkers may improve the performance of individual tests. Should a combination of genomic, metabolomic, and proteomic urinary biomarkers be more robust and predictive of long-term outcomes, the ExoTRU test may have utility in serial measurements of posttransplant renal function, thereby providing background for future clinical studies and guidance for immunosuppressive therapies.

We identified a signature to differentiate ABMR from TCMR that included 4 of 5 genes (*CXCL11*, *CD74*, *CD44*, and *C3*) previously identified, demonstrating the generalizability of these markers in a novel assay format. In addition, we identified *IL18BP* (encoding IL18 binding protein) as a strong predictor of rejection. Of all 5 genes, *IL18BP* had the best AUC of any single marker (0.77), which is an inhibitor of the proinflammatory cytokine IL18 that has been shown to enhance Th1 responses implicated in kidney transplant rejection.[39] Moreover, IL18BP transgenic mice are protected from ischemic acute kidney injury, and IL18BP therapy is protective in Adriamycin nephropathy.[40]

The strength of this urinary exosome mRNA study is that it further elucidated a set of biomarkers associated with active renal allograft rejection and significant kidney inflammation in a large number of patients and urine samples, and described a signature that is useful as a rule-out test to avoid unnecessary biopsies. In addition, it demonstrated good prognostic performance, because samples with positive signatures were statistically associated with a higher risk of 5-year adverse outcomes, and will thus help improve clinical management. This signature has high generalizability and standardization characteristics, which remain important when developing diagnostic or prognostic tests.

This study has 2 limitations. First, this was a cross-sectional study, and serial urine samples were not collected, precluding the analysis of how early the signature could predict rejection before clinical indication. However, preliminary analysis of long-term allograft outcomes demonstrated the potential of this signature to predict future adverse events, including rejection. Second, given the lack of longitudinal samples collected from stable patients, the natural variation in the performance of the urine exosome mRNA classifiers could not be determined.

In conclusion, the gene signatures described herein may provide clinicians with a tool for detecting earlystage renal allograft rejection and for risk

stratification in kidney transplant recipients. Biomarkers offer insights into the molecular mechanisms underlying posttransplant allograft dysfunction, such as immune cell activation and proinflammatory cascades. A larger validation study with prospectively collected samples would further confirm our results and permit the earlier detection of subclinical injury and acute rejection.

DISCLOSURE

JRA, JH, EH, BCH, and JS have intellectual properties related to this study. JRA reports having intellectual properties and receiving royalties from Accrue Health Inc.; receiving research funding from ExsosomeDx, CareDx, Moderna Inc., Alexion Therapeutic; being a scientific advisor for CareDx. KF, JH, BCH, EH, SX, SK are employees of Exosome Diagnostics, a Bio-Techne brand. RF reports having consultancy agreements with Genentech Pharmaceutical and Veloxis Pharmaceuticals; being on a speakers' bureau for Novartis Pharmaceuticals; being on the visiting committee for the Scientific Registry of Transplant Recipients (January 1, 2018); and being a member of the United Network for Organ Sharing/Organ Procurement and Transplantation Network Membership and Professional Standards Committee. LVR reports receiving research funding from Bristol Myers Squibb and Visterra and being a scientific advisor for or member of CareDx. JS reports ownership interest in biotechnology, patents, and inventions with Massachusetts General Hospital. All the other authors declared no competing interests.

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DATA AVAILABILITY STATEMENT

The datasets analyzed in all reported studies may be available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS

JS and JRA contributed to the conception and design. REF, AA, JC, ZS, CD, AS, AH, NY, KC, MM, CV, and AV contributed to data acquisition. KF, JH, BCH, EH, SX, SK performed assays and data analysis. REF, BCH, and JRA guided the initial draft of the manuscript. All the authors contributed to data interpretation and manuscript revisions; and approved the final version of the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary material (pdf)

Supplementary Methods.

Figure S1. Scatterplot analysis of the performance of in-house designed assays versus the commercial assays used in the original study.

Figure S2. CONSORT flow diagram and histological diagnosis matching the urine samples included in the analysis.

Figure S3. For-cause classifier performance, segmented by race, demonstrated no significant differences in the performance of the urinary exosomal mRNA signature by ROCcurve analysis.

Figure S4. Urinary exosomal for-cause biopsy signature (ExoTRU) score between nonrejection cases and rejection subtypes.

Figure S5. Waterfall analysis of urinary exosomal signatures with for-cause samples, assessing varying degrees of inflammation.

Table S1. Urinary exosomal for-cause biopsy signature demonstrates strong rule-out performance, correctly classifying 57 of the 61 any-cause rejection biopsies.

Table S2. Significant inflammation categories: samples matched with kidney biopsy with significant inflammation according to the pathology report.

Table S3. Adverse events overview: adverse events include $\geq$ 1 episode of rejection that happened within 5 years from urine sample collection, a persistent $>$ 30% decrease eGFR, from baseline.

STROBE checklist.

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